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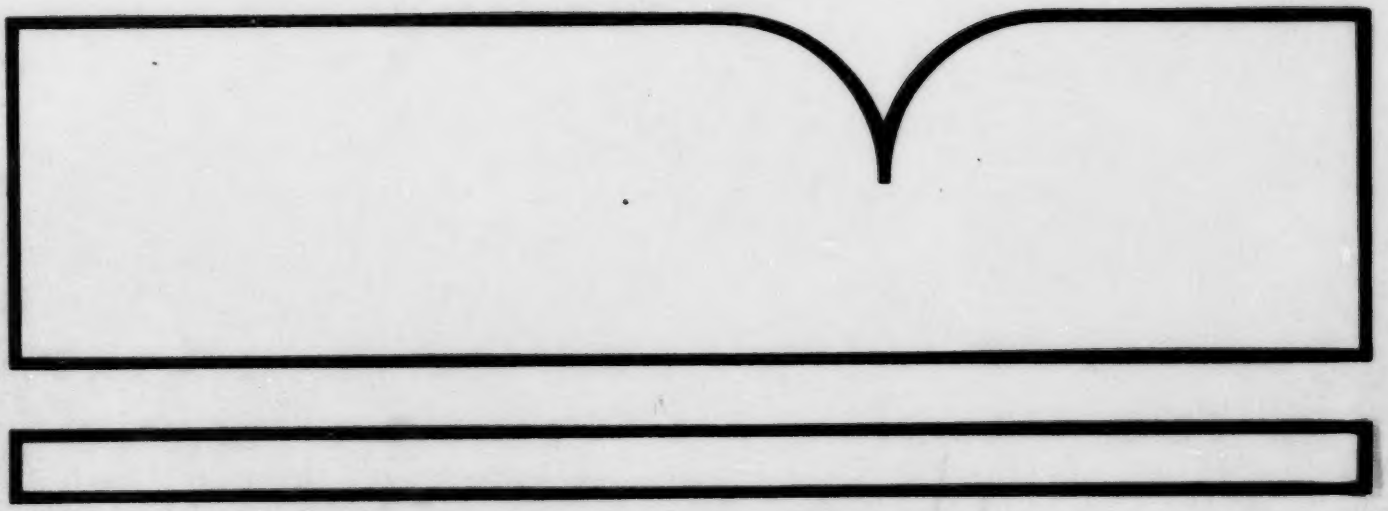

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ASSESSMENT OF THE TLC/SALMONELLA ASSAY FOR SCREENING HAZARDOUS WASTES

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ABSTRACT

Using a modified version of the TLC/Salmonella assay developed by Bjørseth et al. (1982), ten complex hazardous wastes were tested for mutagenic activity. This method couples thin layer chromatography (TLC) with the Salmonella/mammalian-microsome (Ames) assay for the detection of mutagenic constituents in complex mixtures. Crude hazardous wastes and selected hazardous waste extracts were fractionated on commercially available cellulose TLC plates. Mutagenicity testing was performed by applying a single overlay of minimal growth agar containing a tester strain of Salmonella and the optional metabolic activation system directly onto the developed chromatogram. The appearance of localized clusters of revertant colonies or an increase in total revertant growth vis-a-vis control plates indicated a mutagenic response. Seven of ten hazardous wastes demonstrated mutagenic activity when tested by this method.

To assess the sensitivity of the modified TLC/Salmonella assay, fourteen Salmonella mutagens from a wide range of chemical classes and polarities were tested. The selected compounds included heterocyclics, aromatic amines, alkylating agents, antitumor agents, a nitrosamine, and a nitroaromatic. Eleven of the fourteen mutagens were positive in this test system. The three compounds refractory to analysis included a polycyclic aromatic hydrocarbon and two volatile compounds.

INTRODUCTION

Complex hazardous wastes are composed of compounds that manifest diverse chemical, physical, and toxicological properties. Human exposures to hazardous wastes have been associated with a number of adverse health effects, including chromosomal damage, cancer, and reproductive anomalies (Maugh, 1979; Vianna and Polan, 1984; Warren, 1981). Adequate characterization of the genotoxic potential of hazardous wastes is therefore crucial to the assessment of risk to human health.

Assessments of genotoxic risk are based in part upon results from short-term in vitro assays. One such test, the Salmonella/mammalian-microsome assay (Ames et al., 1975; Maron and Ames, 1983) has been used for over a decade to predict the mutagenic potential of pure compounds and complex mixtures. To enhance detection capabilities, in vitro assays are often coupled with chemical or physical fractionation techniques to separate or remove toxic components from the complex sample prior to testing and to reduce the effects of interactive mechanisms. For example, in a study performed by Bjørseth et al. (1982), thin layer chromatography was used to segregate potential mutagens in a complex environmental mixture, and the Salmonella assay was then applied directly onto the developed chromatogram. The subsequent appearance of localized clusters of revertant colonies suggested the presence of a mutagenic constituent. This assay has been successfully applied to the analysis of air samples (Bjørseth et al., 1982), extracts of typewriter ribbons and carbon paper (Bjørseth et al., 1982; Møller et al., 1983), and emission samples from coal- and oil-fired boilers (Alfheim et al., 1983).

We modified the TLC/Salmonella assay described by Bjørseth and his colleagues by replacing the double-agar system with a single overlay

composed of agar, tester strain, and the metabolic activation system. This modification simplified the technique, reduced preparation time, and, additionally, provided direct contact between the bacteria and partitioned components on the chromatogram, ostensibly enhancing detection of nonpolar and intermediately polar compounds. A complete description of this work has been published elsewhere (Houk and Claxton, 1986).

Ten hazardous wastes were screened for mutagenic activity using this modified assay. Samples were selected from a variety of dissimilar waste types, including acids, caustics, tars, emulsions, and sludges. Solids, semi-solids, and liquids were represented, as were inorganic and organic wastes.

The detection capabilities of this modified TLC/Salmonella assay were challenged with fourteen compounds known to be mutagenic in the Salmonella plate incorporation assay. Mutagens were selected from a wide range of chemical classes and polarities, and included alkylating agents, aromatic amines, antitumor agents, a nitrosamine, and a nitroaromatic.

MATERIALS AND METHODS

The hazardous waste samples evaluated in this study were obtained from Batelle Columbus Laboratories, Columbus, Ohio, courtesy of Dr. M. McKown. Table 1 provides gross chemical and physical characterizations and a cursory description of each waste tested. Results from gas chromatography/mass spectrometry (GC/MS), emission spectroscopy, and analysis for purgeable and semi-volatile organic compounds have been published elsewhere (Battelle, 1981; Houk, 1984; Miller et al., 1981; Warner et al., 1981).

Most samples arrived in physical states that favored direct application to TLC plates (liquids, emulsions, sludges, and a tar). The organic still bottoms and the dewatered municipal sludge, however, were

solids and could not be applied to plates in their unadulterated states. Ethanol (ETOH) extracts of these wastes were tested. Ethanol and dimethyl sulfoxide (DMSO) extracts of the coke plant waste were also obtained and were tested in addition to the crude sample. Extractions were performed as previously described (Andon et al., 1985).

The TLC/Salmonella technique was a modification of the procedure developed by Bjørseth et al. (1982) and is described in detail in Houk and Claxton (1986). The TLC separations were performed on commercially available glass-backed cellulose plates (100 mm x 100 mm). Neat samples or extracts were spotted on the plates at an origin 15 mm from the bottom edge and 20 mm apart. Doses ranged from approximately 0.1 to 16 ul per application point. Plates were developed at room temperature in chloroform for 15 to 20 min, at which time the solvent front had ascended to approximately 10 mm from the top edge of the plate. When chloroform proved ineffective in fractionating the sample, other carrier systems were utilized (see Results and Discussion).

After the plates were removed from the developing chamber and the mobile phase had evaporated from the plate, developed plates were inspected for chromatographic patterns using ambient and ultraviolet light sources. Chromatograms were placed sorbent side up into over-sized Petri dishes (150 mm by 15 mm) for mutagenicity testing. Genotoxic analyses were performed in situ. A mixture containing agar, minimal growth medium, the bacterial strain, and the optional metabolic activation system was poured over the TLC plates. The Petri dishes were incubated for 72 h at 37°C.

Salmonella typhimurium strains TA98 and TA100 were obtained from Dr. Bruce Ames. Rat liver homogenate (S9) was prepared from rats injected with Aroclor 1254 as previously described (Ames et al., 1975). Developed blank TLC plates were used as negative controls. Undeveloped plates spotted with 2-anthramine,

2-nitrofluorene, or sodium azide were used as positive controls.

After incubation, the plates were examined for toxicity, as evidenced by (1) a reduction in the overall number of revertants when compared to spontaneous values or (2) a discrete toxic zone devoid of bacterial growth. A mutagenic event was indicated by the appearance of one or more localized clusters of histidine revertants or as a reproducible twofold or greater increase in revertant colonies (for the total plate) over spontaneous background values. Dose-response relationships were examined by applying four different volumes per plate. Independent experiments were performed to guarantee reproducibility and to confirm positive or negative findings. Results were documented by tracing observed chromatographic patterns and revertant colony locations onto transparent sheets of cellulose acetate.

To examine the predictive capabilities of the assay, fourteen Salmonella mutagens of various classes and polarities were selected for testing. Increasing volumes of the mutagen solution were spotted in clockwise fashion at the four corners of a cellulose plate. Plates were not chromatographed, but were placed undeveloped into Petri dishes and tested for mutagenic response using the single overlay previously described.

RESULTS AND DISCUSSION

Analysis of Hazardous Wastes

Results from the mutagenicity testing of the hazardous waste samples are given in Table 2.

ORGANIC SLUDGE

A relatively nonpolar mutagenic component migrated to a high R_f value following chromatography of the organic sludge sample. Figure 1a illustrates

the observed mutagenic response when the chromatogram was tested with TA98 in the absence of metabolic activation.

The diluted counterpart of this sludge, spiked with eleven organic compounds, failed to demonstrate similar activity (Table 2). Two explanations are plausible. The organic compounds which were added to the original sludge could have interfered with the mutagenic expression of the resulting sample. On the other hand, diluting the parent sludge may have masked the original activity, permitting mutagenic expression of the added compounds.

EDC SPENT CAUSTIC

The EDC spent caustic contained a mutagen(s) that induced a dose-dependent cluster of revertant colonies in both strains without metabolic activation. Activity was localized around fluorescent orange bands that migrated with the solvent front (Figure 1b). The migration of this direct-acting mutagen suggests that the compound is relatively nonpolar.

HERBICIDE MANUFACTURING ACID

With activation, the herbicide manufacturing acid produced intense clustering of revertant colonies around the points of sample application (both strains). Clustering was localized within circular fluorescent zones (Figure 1c). At doses greater than 1 ul, a zone of toxicity surrounded by a ring of revertant colonies was evident. These results indicate that indirect-acting mutagen(s) are present that are toxic to Salmonella when administered at sample doses greater than 1 ul.

The mutagenic constituent present in this herbicide waste was not desorbed by chloroform and consequently did not ascend the TLC plate. Thus, two nonpolar mobile phases described for pesticide separations were

selected to try to enhance migration. The first was a solvent system for separation of chlorinated pesticides (heptane-acetone, 98:2) described by Stephens and Chan (1980). The second was a system that has proven useful in the separation of organophosphorus pesticides (hexane-acetone, 5:1) (Stephens and deVera, 1979). Both solvent systems failed to partition the herbicide waste. A polar mobile phase (dichloromethane/ethanol/water, 10:20:1) described by Tomingas et al. (1977) subsequently proved successful in desorbing the mutagenic compound(s). Most revertant activity remained localized midway up the plate within the fluorescent zone (Figure 2).

The initial localization of revertant activity around the points of sample application suggests that the mutagen(s) is very polar. Changes in the mobile phase system from nonpolar to polar have supported this assumption. These findings demonstrate the usefulness of testing different solvent systems to more clearly define chemical properties.

COKE PLANT WASTE

The neat coke plant waste produced approximately a two-fold increase in the number of spontaneous His^+ revertants when tested with TA98 in the absence of exogenous activation. Ethanol and DMSO extracts of this sample produced clusters of colonies at points of sample application (figures not included). Independent analysis using the standard plate incorporation assay supports our findings of mutagenic activity in the extracts (Andon et al., 1985). Additional verification of the mutagenic potential of this coke plant waste comes from GC/MS analysis, which revealed the presence of the mutagens/carcinogens benzo(a) pyrene (1.12 ug/g), benzo(b) and benzo(k)fluoranthene (0.69 ug/g), and chrysene and benzo(a)anthracene (0.5 ug/g) (Miller et al., 1981).

INK PIGMENT SLUDGE

No mutagenic activity was evident when ink pigment sludge was tested with TA98. With TA100, results proved inconclusive. Three independent experiments were performed with increasing dosages, but convincing results could not be obtained.

LATEX PAINT WASTE

Results from the TLC/Salmonella assay of the latex paint waste were negative. However, elemental analysis of this waste indicated the presence of several carcinogenic and mutagenic metals, including cadmium, arsenic, and selenium (Miller et al., 1981). In general, the Salmonella reversion assay does not detect mutagenic metals without bioassay modifications, so it follows that the TLC/Salmonella assay would also prove unsuitable for the routine screening of metal-containing wastes.

COAL GASIFICATION TAR

The coal gasification tar was too viscous to be drawn into a pipette tip. Instead, the tip was touched onto the surface of the sticky substance and a small amount of tar was transferred to the TLC plate. Consequently, applied sample volumes are unknown.

When chromatographed, this sample streaked the length of the plate with no definition of component parts. Mutagenicity testing suggests that this complex sample contains indirect-acting frameshift mutagens that are not particularly water-soluble. This conclusion is based upon the fact that TA98 (+S9) revertants are localized along the length of the visualized streak and are not profuse across the plate (Figure 1d). There also appear to be direct-acting frameshift mutagens that are more water-soluble, as evidenced by the diffuse response observed on plates without metabolic activation (figure not shown).

ORGANIC STILL BOTTOMS, ETOH EXTRACT

There was no evidence of mutagenic or toxic activity in either strain.

DEWATERED MUNICIPAL SLUDGE, ETOH EXTRACT

Revertant clustering was observed in the area of the origin when the chromatogram of the municipal sludge was tested with metabolic activation (both strains). These results suggest the presence of indirect-acting mutagens that are polar and probably water-soluble.

OIL REFINING WASTE

In independent duplicate experiments there appeared a slight dose-response clustering around the origin in TA98 with activation. This effect was evident only at low doses (from 0.5 to 2 ul) and disappeared at volumes of 4 ul and greater. The explanation for this response may be linked to the influence of a fluorescent spot that became larger as sample volumes increased. This separated component appeared to be toxic to the bacteria. An overall reduction of colony counts vis-a-vis control plates was also noted.

Supportive evidence of this sample's toxic potential comes from a study using the CHO cytotoxicity test (Andon et al., 1985). This sample produced a greater than 50% reduction both in the cellular ATP concentration and in the viability index in Chinese hamster ovary cells. Because oil refining wastes have been shown to contain significant levels of lead and chromium (U.S. Environmental Protection Agency, 1980), the observed cytotoxic response may ostensibly be linked to these toxic metals.

Analysis of Model Compounds

Results from the testing of the Salmonella mutagens are given in Table 3. Eleven of the fourteen test compounds were positive. Three compounds --benzo(a)pyrene (BaP), epichlorohydrin, and dimethylcarbamyl

chloride--registered negative responses, even at very high dose levels (Table 3). Previous TLC/Salmonella research on BaP also resulted in negative findings (Bjørseth et al., 1982). As reported by Alfheim et al. (1983) and Møller et al. (1983), this assay system appears unsuitable for the detection of non-polar organics such as unsubstituted PAHs.

The lack of demonstrable mutagenic activity shown by epichlorohydrin and dimethylcarbamyl chloride is presumably linked to their high volatility. These unstable compounds probably volatilized from the chromatography plate prior to testing, thereby precluding detection by the mutagenesis assay.

A nonmutagen (1-naphthylamine, 2-80 ug) was also tested and did not register a mutagenic response.

CONCLUSION

Our findings demonstrate that the TLC/Salmonella assay described in this paper can detect mutagens in complex hazardous wastes. Of ten wastes tested, seven were found to contain mutagenically active compounds. The assay proved to be applicable to a broad range of chemically and physically dissimilar waste types. Liquid or near-liquid samples could be applied to the TLC plates without preparatory workup. Solid wastes, however, had to be converted to a form suitable for TLC application and development. The system proved sensitive to microliter volumes of crude test sample.

Additionally, our investigation has shown that detection capabilities cover a wide range of mutagen classes and polarities. Mutagenic activity was detectable in the microgram range. Compounds refractory to analysis by the TLC/Salmonella assay included two mutagens that were volatile and an unsubstituted PAH, a class that does not ordinarily evoke a mutagenic response in a spot test.

Chemical and physical properties of detected mutagens may be inferred from parameters such as localization on the TLC plate (expressed as an R_f value), polarity of the mobile phase, and response to visualization techniques (fluorescence, color, etc.). Fluorescent mutagens were present in the organic sludge, the EDC spent caustic, the herbicide manufacturing acid, and the coal gasification tar. Polar mutagens (those with low R_f values) were detected in the herbicide manufacturing acid, the municipal sludge, and the oil refining waste, while nonpolar mutagens were detected in the organic sludge and the EDC spent caustic.

The TLC/Salmonella assay offers several important advantages over other coupled fractionation/bioassay schemes. The procedure is very rapid. Fractionation of the samples by chromatography can be completed within 10 to 20 minutes. Mutagenicity results are documented after 72 hours. Samples can be applied to the system in their neat state; thus, extraction procedures are obviated except for samples that cannot be chromatographed in their crude form (e.g., solids). Compounds that may exert synergistic or antagonistic effects are separated from the complex sample during chromatography and are detected independently. Problems with sample toxicity are minimized.

In summary, the TLC/Salmonella assay represents a simple screening tool for the qualitative detection of mutagens in complex environmental samples. The speed and economy with which results can be obtained make it useful for routine testing and for processing large numbers of samples. The presumptive identification of mutagenic constituents in a sample can lead to a systematic selection of that sample for more conclusive chemical or biological evaluation.

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TABLE 1

DESCRIPTION OF HAZARDOUS WASTE SAMPLES

Waste	Physical state	pH [a]	Total solids [a] (%)	Description
Organic sludge	Liquid	NM	NM	Sludge from an industrial solvent recovery process
Organic sludge, spiked	Liquid	NM	NM	Sludge described above, diluted and fortified with 11 organics
EDC spent caustic (water portion)	Liquid	5.2	<1.0	Clear, tan liquid waste from ethylene dichloride production
Herbicide mfg. acid	Liquid	0.5	42	Thin, gray aqueous suspension from an herbicide production process
Coke plant waste	Liquid	8.0	4	Thin, brown fluid with suspended solids, 95% DMSO-soluble, water-insoluble
Ink pigment sludge	Semi-solid	8.7	14	Pourable black jelly from ink production
Latex paint mfg. waste	Suspension	7.5	41	Thick polymeric emulsion of pigment, 50% soluble in DMSO
Coal gasification tar	Tar	8.1	79	Black, viscous tar produced during coal gasification
Organic still bottoms	Solid	3.8	83	Thick, black slurry from tank bottoms
Dewatered municipal sludge	Solid	7.5	44	Dark, wet cake of chemically treated municipal sludge
Oil-refining waste	Semi-solid	7.0	59	Dark liquid with black flocculent and oil droplets, DMSO- and water-soluble

[a] Analysis performed by Research Triangle Institute (Sparacino, 1983).
NM, not measured.

TABLE 2

RESULTS FROM THE TLC/SALMONELLA ANALYSIS OF HAZARDOUS WASTES

Waste	Extract [a]	TLC plate dose range (μl/application)	Mutagenicity			
			TA98		TA100	
			+S9	−S9	+S9	−S9
Organic sludge	NEAT	0.25–2	+ [b]	+ [c]	+ [d]	−
Organic sludge, spiked	NEAT	0.25–16	−	+ [d]	−	−
EDC spent caustic	NEAT	0.5–8	−	+ [c]	−	+ [b,c]
Herbicide mfg. acid	NEAT	0.1–4	+ [b,c]	−	+ [b,c]	−
Coke plant waste	NEAT	0.5–10	−	+ [b]	−	−
	EtOH	0.5–6	+ [c]	−	−	−
	DMSO	0.5–8	−	−	−	+ [d]
Ink pigment sludge	NEAT	0.5–4	−	−	incl	incl
Latex paint mfg. waste	NEAT	0.5–12	−	−	−	−
Coal gasification tar	NEAT	unknown	+ [b,c]	+ [b]	+ [d]	−
Organic still bottoms	EtOH	0.1–8	−	−	−	−
Dewatered municipal sludge	EtOH	1–16	+ [d]	−	+ [d]	−
Oil-refining waste	NEAT	0.5–4	+ [d]	T	−	−

[a] Extract: NEAT, unadulterated; EtOH, ethanol; DMSO, dimethyl sulfoxide.
[b] 2-fold or greater increase in revertant counts (for the total plate) over spontaneous background values.
[c] Clustering of colonies; distinct localization.
[d] Clustering of colonies; localization detected by grid analysis.
incl, inconclusive findings.
T, toxic.

TABLE 3

RESULTS FROM TLC/SALMONELLA ANALYSIS OF MODEL COMPOUNDS

Compound	Responsive dose range [a] (μg/application)	Mutagenic activity		
		Result	Strain	Activation
Sodium azide	2–16	+ [b]	TA100	−S9
MMS	2–16	+ [b]	TA100	−S9
β-Propiolactone	100–400	+ [b]	TA100	−S9
2-Nitrofluorene	2–16	+ [c]	TA98	−S9
Cyclophosphamide	2–16	weak + [c]	TA100	+S9
	20–80	+ [c]	TA100	+S9
2-AAF	2–16	+ [c]	TA98	+S9
	20–80	+ [c]	TA100	+S9
Daunomycin	0.125–1	+ [c]	TA98	−S9
	2–16	toxic	TA98	−S9
Streptozotocin	2–16	+ [d]	TA100	−S9
4-NQO	0.5–16	+ [d]	TA98	−S9
	0.5–16	+ [d]	TA100	−S9
MNNG	2–16	+ [d]	TA100	−S9
2-Anthramine	2–16	+ [d]	TA98	+S9
	2–16	+ [d]	TA100	+S9
Benzo[*a*]pyrene	2–80	−	TA98	+S9
	2–80	−	TA100	+S9
Epichlorohydrin	2–200	−	TA100	−S9
Dimethylcarbamyl chloride	2–2000	−	TA100	−S9

[a] Responsive dose range does not necessarily reflect the lowest possible or highest achievable dose. It merely indicates those doses tested.
[b] Profusion of revertant colonies across the plate.
[c] Localized clusters of revertant colonies at points of sample application.
[d] Dose-dependent ring of revertant colonies around a central toxic zone.

FIGURE 1: SEPARATION OF MUTAGENIC COMPOUNDS BY THE TLC/SALMONELLA ASSAY.

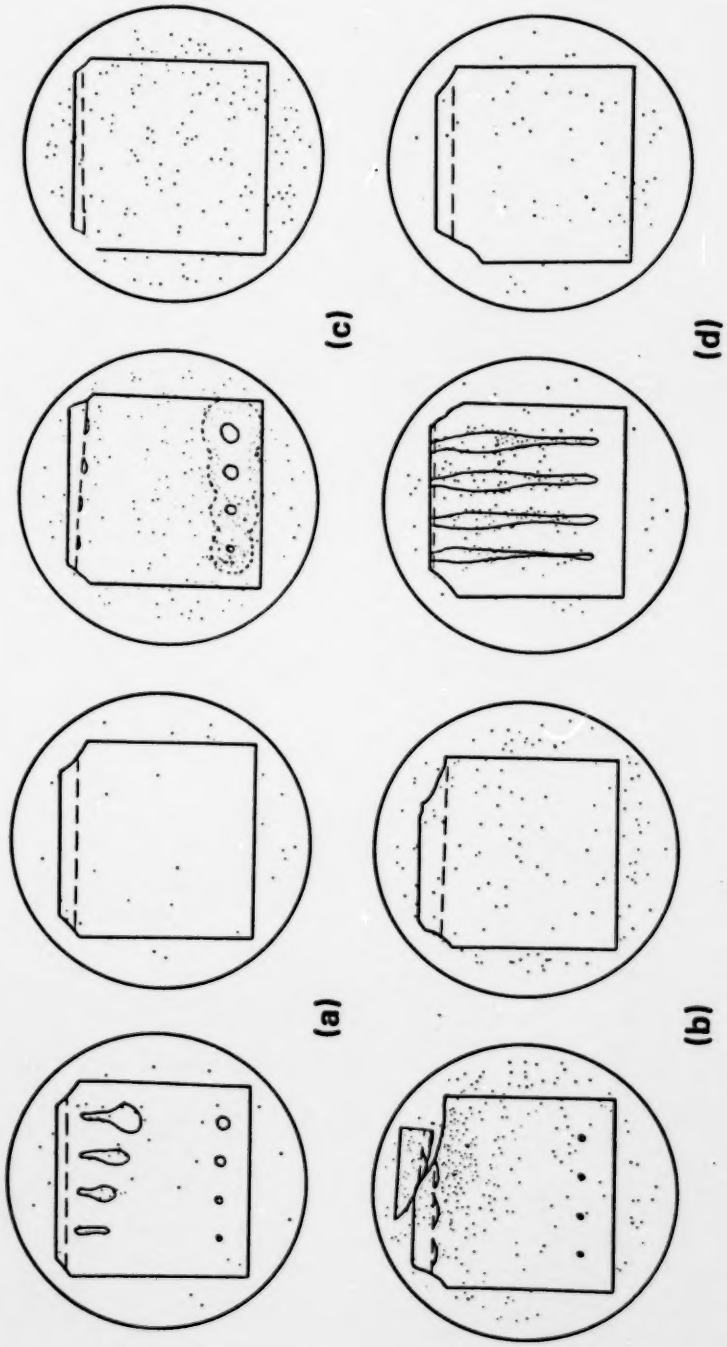
Samples were spotted at increasing volumes across the plate, chromatographed with chloroform, and tested for mutagenic activity. Sample application points and chromatographed spots are indicated (see text for discussion). Negative control plates (spontaneous revertants) are also illustrated. o, application point; -----, solvent front.

(a) Organic Sludge (0.25, 0.5, 1.0, 2.0 ul) tested with TA98, -S9.

(b) EDC Spent Caustic (2, 4, 6, 8 ul) tested with TA100, -S9. Plate was broken during excision of top corners.

(c) Herbicide Manufacturing Acid (0.5, 1.0, 2.0, 4.0 ul) tested with TA100, +S9.

(d) Coal Gasification Tar (unknown volumes) tested with TA98, +S9.


(c)
(d)
(a)
(b)

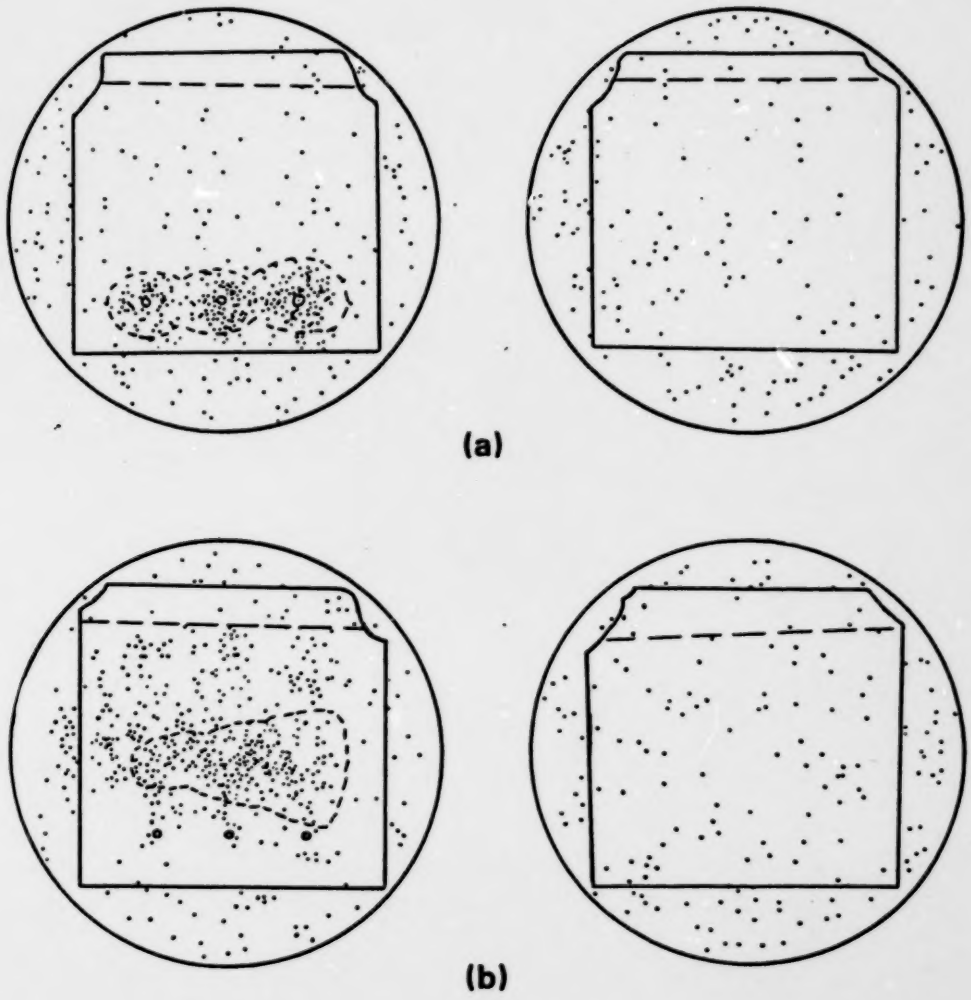


FIGURE 2: TLC/SALMONELLA ANALYSIS OF THE HERBICIDE MANUFACTURING ACID CHROMATOGRAPHED WITH DIFFERENT SOLVENT SYSTEMS

Fluorescent zones (denoted by a dashed line) are located around sample application points in (a) and midway up the plate in (b). Negative control plates (spontaneous revertants) are also illustrated.

(a) Sample Volumes: 0.1, 0.25, 0.5 ul
Mobile Phase: Chloroform
Mutagenicity Testing: TA100 with activation

(b) Sample Volumes: 0.1, 0.25, 0.5 ul
Mobile Phase: DCM/ETOH/Water, 10:20:1
Mutagenicity Testing: TA100 with activation

o, application point; - - - -, solvent front.